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PROPHYLAXIS AND TREATMENT OF SEPSIS AND SEPSIS-ASSOCIATED COAGULATION DISORDERS

Abstract:

A method is shown for using LACI wherein a therapeutically-effective amount thereof is administered to a patient afflicted with or susceptible to sepsis.

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PROPHYLAXIS AND TREATMENT OF SEPSIS AND SEPSIS-ASSOCIATED COAGULATION DISORDERS

RELATED APPLICATION

This application is a continuation-in-part of U.S. Application Serial No. 07/897,135 filed 11 June 1992.

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

The present invention relates to prophylaxis and treatment of sepsis and sepsis-associated coagulation disorders such as disseminated intravascular coagulation (DIC) due to sepsis. More particularly, the present invention relates to prophylaxis and treatment of sepsis, particularly gram negative sepsis, or sepsis-associated disorders, such as DIC due to sepsis, by administering a therapeutically-effective amount of lipoprotein-associated coagulation inhibitor (LACI) to a patient afflicted with or susceptible to sepsis or sepsis-associated disorders.

2. BACKGROUND INFORMATION

Lipoprotein-associated coagulation inhibitor (LACI) is a protein inhibitor present in mammalian blood plasma. LACI is also known as tissue factor (TF) inhibitor, tissue thromboplastin (Factor III) inhibitor, extrinsic pathway inhibitor (EPI) and tissue factor pathway inhibitor (TFPI).

Blood coagulation means the conversion of fluid blood to a solid gel or clot. The main event is the conversion of soluble fibrinogen to insoluble strands of fibrin, although fibrin itself forms only 0.15% of the total blood clot. This conversion is the last step in a complex enzyme cascade. The components (factors) are present as zymogens, inactive precursors of

proteolytic enzymes, which are converted into active enzymes by proteolytic cleavage at specific sites. Activation of a small amount of one factor catalyses the formation of larger amounts of the next, and so on, giving an amplification which results in an extremely rapid formation of fibrin.

The coagulation cascade which occurs in mammalian blood is divided by in vitro methods into an intrinsic system (all factors present in the blood) and an extrinsic system which depends on the addition of thromboplastin. The intrinsic pathway commences when the first zymogen, factor XII or 'Hageman Factor', adheres to a negatively charged surface and in the presence of high molecular weight kininogen and prekallikrein, becomes an active enzyme, designated XIIa. The activating surface may be collagen which is exposed by tissue injury. Factor XIIa activates factor XI to give XIa, factor XIa activates factor IX to IXa and this, in the presence of calcium ions, a negatively charged phospholipid surface and factor VIIIa, activates factor X. The negatively charged phospholipid surface is provided by platelets and in vivo this serves to localize the process of coagulation to sites of platelet deposition. Factor Xa, in the presence of calcium ions, a platelet-derived negatively charged phospholipid surface and a binding protein, factor V, activates prothrombin to give thrombin (IIa) - the main enzyme of the cascade. Thrombin, acting on gly-arg bonds, removes small fibrinopeptides from the N-terminal regions of the large dimeric fibrinogen molecules, enabling them to polymerize to form strands of fibrin. Thrombin also activates the fibrin stabilizing factor, factor XIII, to give XIIIa, a fibrinoligase, which, in the presence of calcium ions strengthens the fibrin-to-fibrin links with intermolecular γ-glutamyl-ξ-lysine bridges. In addition, thrombin acts directly on platelets to cause aggregation, and release of subcellular constituents and arachidonic acid. A further function of thrombin is to activate the coagulation inhibitor, protein C. Factors XIIa, XIa, IXa, Xa, and thrombin are all serine proteases.

The extrinsic pathway in vivo is initiated by a substance generated by, or exposed by, tissue damage and termed 'tissue factor', interacting with Factor VII in the presence of calcium ions and phospholipid to activate factors X and IX, after which the sequence proceeds as already described. The identity of TF is known. There is evidence that tissue factor occurs in the plasma membranes of perturbed endothelial cells of blood vessels and also in atheromatous plaques.

The two pathways described are not entirely separate because both factor IXa and factor XIIa in the intrinsic pathway may activate factor VII in the extrinsic pathway. There are, in addition, various feedback loops between other factors, which enhance reaction rates. For example, thrombin (IIa) enhances the activation of both factor V and factor VIII.

Sepsis is a toxic condition resulting from the spread of bacteria, or their products (collectively referred to herein as bacterial endotoxins) from a focus of infection. Septicemia is a form of sepsis, and more particularly is a toxic condition resulting from invasion of the blood stream by bacterial endotoxins from a focus of infection. Sepsis can cause shock in many ways, some related to the primary focus of infection and some related to the systemic effects of the bacterial endotoxins. For example, in septacemia, bacterial endotoxins, along with other cell-derived materials, such as IL-1, IL-6 and TNF, activate the coagulation system and initiate platelet aggregation. The process leads to blood clotting, a drop in blood pressure and finally kidney, heart and lung failure.

Activation of the coagulation cascade by bacterial endotoxins introduced directly into the bloodstream can result in extensive fibrin deposition on arterial surfaces with depletion of fibrinogen, prothrombin, factors V and VIII, and platelets. In addition, the fibrinolytic system is stimulated, resulting in further formation of fibrin degradation products. Disseminated intravascular coagulation (DIC) is a complex

coagulation disorder resulting from widespread activation of the clotting mechanism or coagulation cascade which, in turn, results from septicemia. Essentially, the process represents conversion of plasma to serum within the circulation system. Such process represents one of the most serious acquired coagulation disorders.

Attempts to treat DIC due to septicemia utilizing heparin have been made. See, for example, Corrigan et al., "Heparin Therapy in Septacemia with Disseminated Intravascular Coagulation. Effect on Mortality and on Correction of Hemostatic Defects", N. Engl. J. Med., 283, 778-782 (1970); Lasch et al., Heparin Therapy of Diffuse Intravascular Coagulation (DIC)", Thrombos. Diathes. Haemorrh., 33, 105 (1974); Straub, "A Case Against Heparin Therapy of Intravascular Coagulation", Thrombos. Diathes. Haemorrh., 33, 107 (1974).

Day et al [Blood, 76, 1538 (1990)] disclose that LACI inhibits tissue thromboplastin-induced coagulation in the presence of factor X_a. It is suggested that DIC results from inappropriate exposure of tissue factor to plasma.

Wun et al, EP 473,564, discloses that a combination of LACI and heparin can be used to inhibit both the intrinsic and extrinsic pathways of coagulation and therefore may be effective for treatment of DIC.

Nordfang [WO91/19514, published Dec. 26, 1991] discloses combining heparin with EPI and suggests that such combination would be effective to treat coagulation disorders or cancer.

Taylor et al. [Blood, 78, 364 (1991)] suggest a factor X_a derivative for treatment of DIC due to sepsis. Such derivative was prepared by blocking factor X_a in the active center with [5-(dimethylamino)1-naphthalene-sulfonyl]-

glutamylglycylarginyl chloromethyl ketone. However, the inflammatory, coagulant, and cell injury responses to E. coli of both the treated and control groups were lethal.

BRIEF DESCRIPTION OF THE INVENTION

It has now been discovered that LACI in the absence of other anticoagulants such as heparin is effective in the prophylaxis and treatment of sepsis. It has also been discovered that LACI alone is effective in the prophylaxis and treatment of sepsis-associated coagulation disorders such as, for example, DIC. Accordingly, the present invention is directed to a method of using LACI comprising administering LACI to a patient afflicted with or susceptible to sepsis. More particularly, the present invention is directed to a method for prophylaxis or treatment of sepsis or sepsis-associated coagulation disorders comprising administering to a patient in need thereof a therapeutically-effective amount of LACI. A preferred embodiment is a method for treatment or prophylaxis of disseminated intravascular coagulation comprising administering to a patient susceptible to or afflicted with sepsis a therapeutically-effective amount of LACI. A most preferred embodiment is a method for treatment or prophylaxis of sepsis comprising administering to a patient susceptible to or afflicted with sepsis a therapeutically-effective amount of LACI.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a method of using LACI which comprises administering to a patient susceptible to or afflicted with sepsis a therapeutically-effective amount of LACI. In particular, the invention is directed to a method for treatment or prophylaxis of sepsis-associated coagulation disorders.

As utilized herein, the term "sepsis" means a toxic condition resulting from the spread of bacterial endotoxins from a focus of infection.

As utilized herein, the term "sepsis-associated coagulation disorder" means a disorder resulting from or associated with coagulation system activation by a bacterial endotoxin, a product of such bacterial endotoxin or both. An example of such sepsis-associated coagulation disorder is disseminated intravascular coagulation.

The term "therapeutically-effective amount" as utilized herein means an amount necessary to permit observation of activity in a patient sufficient to alleviate one or more symptoms generally associated with sepsis. Such symptoms include, but are not limited to, death, increased heart rate, and increased respiration, decreased fibrinogen levels, decreased blood pressure, decreased white cell count and decreased platelet count. Preferably, a therapeutically-effective amount is an amount necessary to attenuate a decrease in fibrinogen levels in a patient being treated.

As utilized herein LACI means one or more of the three Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor which are active in treating sepsis. LACI is also known as tissue factor pathway inhibitor (TFPI). This name has been accepted by the International Society on Thrombosis and Hemostasis, June 30, 1991, Amsterdam. TFPI

was first purified from a human hepatoma cell, Hep G2, as described by Broze and Miletich; Proc. Natl. Acad. Sci. USA 84, 1886-1890 (1987), and subsequently from human plasma as reported by Novotny et al., J. Biol. Chem. 264, 18832-18837 (1989); Chang liver and SK hepatoma cells as disclosed by Wun et al., J. Biol. Chem. 265, 16096-16101 (1990). TFPI cDNA have been isolated from placental and endothelial cDNA libraries as described by Wun et al., J. Biol. Chem. 263, 6001-6004 (1988); Girard et al., Thromb. Res. 55, 37-50 (1989). The primary amino acid sequence of TFPI, deduced from the cDNA sequence, shows that TFPI contains a highly negatively charged amino-terminus, three tandem Kunitz-type inhibitory domains, and a highly positively charged carboxyl terminus. The first Kunitz-domain of TFPI is needed for the inhibition of the factor VII_a/tissue factor complex and the second Kunitz-domain of TFPI is responsible for the inhibition of factor X_a according to Girard et al., Nature 328, 518-520 (1989), while the function of the third Kunitz-domain remains unknown. See also U.S. 5,106,833. TFPI is believed to function in vivo to limit the initiation of coagulation by forming an inert, quaternary factor X_a: TFPI: factor VII_a: tissue factor complex. Further background information on TFPI can be had by reference to the recent reviews by Rapaport, Blood 73, 359-365 (1989); Broze et al., Biochemistry 29, 7539-7546 (1990).

Recombinant TFPI has been expressed as a glycosylated protein using mammalian cell hosts including mouse C127 cells as disclosed by Day et al., Blood 76, 1538-1545 (1990), baby hamster kidney cells as reported by Pedersen et al., J. Biol. Chem. 265, 16786-16793 (1990), Chinese hamster ovary cells and human SK hepatoma cells. The C127 TFPI has been used in animal studies and shown to be effective in the inhibition of tissue factor-induced intravascular coagulation in rabbits according to Day et al., supra, and in the prevention of arterial reocclusion after thrombolysis in dogs as described by Haskel et al., Circulation 84, 821-827 (1991).

Recombinant TFPI also has been expressed as a non-glycosylated protein using E. coli host cells and obtaining a highly active TFPI by in vitro folding of the protein as described below in Example 1.

The cloning of the TFPI cDNA which encodes the 276 amino acid residue protein of TFPI is further described in Wun et al., U.S. Patent 4,966,852, the disclosure of which is incorporated by reference herein.

Although specific methods of isolation or production of LACI are described herein, it will be understood that the invention is not limited to any particular source of LACI. However, LACI utilized in the present invention is preferably obtained from E. coli as disclosed below in Example 1. Alternatively, LACI can be obtained from cells as disclosed.

The following examples further illustrate the present invention.

EXAMPLE 1

This example illustrates a method for obtaining LACI (TFPI).

MATERIALS

Urea (sequenal grade) and Brij 35 non-ionic surfactant were obtained from Pierce. Mixed bed resin AG501-X8 cation exchanger was purchased from Bio Rad. Mono Q HR 5/5 and HiLoad Q Sepharose anion exchange resins, and Mono S HR 5/5 and Mono S HR 10/16 cation exchange resins were obtained from Pharmacia. Thromboplastin reagent (Simplastin Excel) was from Organon Teknika Corp. Bovine factor X_a and Spectrozyme X_a were supplied by American Diagnostica, Inc.

SDS-PAGE 10-20% gradient gel was obtained from integrated Separation Systems.

METHODS

Expression vectors and cloning strategies

A full length human TFPI cDNA [Wun et al., J. Biol. Chem. 263, 6001-6004 (1988)] was cloned into M13mp18 phage DNA cloning vector as a 1.4 Kb EcoRI fragment. Site-directed mutagenesis [Kunkel et al., Proc. Nat. Acad. Sci. USA 32, 488-492 (1985)] was used to introduce an NcoI site at the initiating ATG. The TFPI gene was then cloned as an NcoI/blunted MaeIII fragment into pMON5557 with NcoI and blunted HindIII ends resulting in the new vector pMON9308. MaeIII site is 15bp downstream from the stop codon in the TFPI cDNA. The expression vector contained the recA promoter, a translational enhancer element and ribosome binding site derived from the gene 10 leader of bacteriophage T7 as described by Olins and Rangwala, J. Biol. Chem. 264, 16973-16976 (1989), and the T7 transcription terminator. This plasmid also contains an irrelevant sequence, i.e. the bST gene (bovine somatotropin).

The NcoI/NsiI fragment of pMON9308 was then replaced by a synthetic DNA fragment designed to (1) introduce an alanine encoding codon at the second position, (2) increase the A-T richness of the 5' portion of the gene, and (3) improve E. coli codon usage. Four oligonucleotides, two for each strand, were used. All base substitutions (indicated in upper case), are silent changes. ECTFPI 2 and 3 were 5' phosphorylated [Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982)]. ECTFPI 1 and 2 and ECTFPI 3 and 4 were annealed in the kinase buffer by incubating for 5 minutes at 70°C and slow-cooling to room temperature. These fragments were cloned into pMON9308 which had been digested with NcoI/NsiI. PCR amplification was used to introduce a HindIII site as well as a TAA termination

codon at the 3' end of the TFPI gene. The PCR primers TPFIterm and TPFIterm 2 are shown below. The TFPI gene was then moved as a NCOI/HindIII fragment into pMON5766. The resultant plasmid was pMON6870.

```
N
c
o          ECTFPI 1
I
catggctgattctgaAgaagatgaagaacaTacTa
    cgactaagactTcttctacttcttgtAtgAtaatagtgA

           ECTFPI 2

                              N
                              s
           ECTFPI 3           i
                              I
ttatcacTgatacTgaACtgccaccGctgaaactGatgca
   ctatgActTGacggtggCgactttgaCt

           ECTFPI 4

                      HindIII
TFPIterm:     ataaca[aagctt]acatatttt

                      NcoI
TFPIterm2:    atatat[ccatgg]ctgattct
```

pMON6870 was digested with BglII/HindIII. This fragment, containing the expression cassette, was cloned into pMON6710 [Obukowicz et al., Biochemistry 29, 9737-9745 (1990)] which had been digested with BglII/HindIII. The resultant plasmid, pMON6875, includes the tac promoter, G10 leader from bacteriophage T7, met-ala TFPI, and the p22 transcriptional terminator. The plasmids were transformed into MON105

(rpoD + rpoH358) containing F' from JM101 for the expression of TFPI protein.

Fermentation

Ten liter fermentations were run in M9 minimal salts media supplemented with 20 g/l casamino acids in Biostad E fermentors (B. Braun). Fermentations were run at a temperature of 37°C, 1000 rpm agitation, an air flow rate of 15 l/min and 10 psi backpressure. pH was controlled at 7.0 with ammonium hydroxide. Residual glucose concentration in the fermentation broth was automatically controlled at 1.0+/-0.1 g/l. At an optical density of 46.0 at 550 nm, the temperature was shifted from 37°C to 30°C and isopropyl ß-D thiogalactopyranoside (IPTG) was added to the fermentor to a final concentration of 1.0 mM. The culture was harvested four hours post-induction by concentration in an Amicon DC10L concentrator followed by centrifugation in a Beckman J2-21 centrifuge. The 10-liter fermentation yield 335-456 g (average of 376+/-46 g, n=6) wet weight of cell paste. The cell paste was frozen at -80°C for further processing hereinbelow.

Isolation of inclusion bodies

Frozen E. coli cell paste was resuspended in cold Milli-Q water at a concentration of 75 g/l. The cells were thoroughly dispersed with a homogenizer (Ultra-Turrax model SD-45) for 30 minutes on ice. The cells were mechanically lysed by three passes through the Manton-Gaulin homogenizer (model 15M-8TA) at 12,000 psi. Inclusion bodies were centrifuged in the Sorvall RC-2B centrifuge in the GSA rotor at 10,000 rpm (16,270 x g) for 20 minutes. The supernatant was discarded. The inclusion body pellets were collected, resuspended in l liter of cold Milli-Q water and dispersed with the Ultra-Turrax homogenizer for 30 minutes on ice. The inclusion bodies were cycled through the Manton-Gaulin homogenizer two more times on ice. Inclusion bodies were

pelleted in the Sorvall RC-2B centrifuge as before. Approximately 60 mg of inclusion bodies were collected for every gram of E. coli cells lysed. The inclusion bodies were stored at -80°C.

Buffer preparation

All the buffers used for sulfonation and refolding of E. coli TFPI contained high concentrations of urea. Urea solutions were treated with Bio-Rad mixed bed resin AG501-X8 at room temperature for at least 20 minutes and filtered through 0.2 µm filter before mixing with buffers. All the solutions used for chromatography were 0.2 µm filtered and sonicated under house vacuum for about 10 minutes.

Sulfonation of inclusion bodies

One gm of inclusion bodies (wet weight) was dispersed in 40 ml of a solution containing 50 mM Tris/HCl, pH 8, and 7.5 M urea by homogenization and vortexing. After the inclusion bodies were largely dissolved, 800 mg of sodium sulfite was added and the mixture was shaken at room temperature for 30 minutes. Then, 400 mg of sodium dithionite or 120 mg of sodium tetrathionate was added and the mixture was shaken at 4°C overnight.. The solution dialyzed against 800 ml of a solution containing 20 mM Tris/HCl, pH 8, and 6 M urea for more than 5 hours at 4°C using a Spectrapor #2 membrane. The dialyzed solution was centrifuged at 48,400 x g for 1 hour, filtered through a 0.2 µm filter, divided into aliquots, and stored at -80°C.

Anion-exchange Chromatography of sulfonated TFPI

On a small scale, the sulfonated and dialyzed inclusion bodies were fractionated on a Mono Q HR5/5 anion exchange column. The column was pre-equilibrated in Q-buffer (20 mM Tris/HCl, pH 8, 6 M urea, 0.01% Brij 35 non-ionic surfactant) containing 0.15 M NaCl. Two ml of sulfonated inclusion bodies were loaded onto the column. The column was washed with 15 ml of the equilibration buffer and eluted with a 30-ml gradient (0.15-0.4 M NaCl) in Q-buffer. Fractions of 1 ml were collected. On a larger scale, 40 ml of sulfonated sample (equivalent to 0.56 g of wet weight inclusion body) was loaded onto a HiLoad Q Sepharose 16/10 anion exchange column pre-equilibrated in Q-buffer containing 0.15 M NaCl. The column was washed with 240 ml of equilibration buffer and then eluted with a 396-ml gradient (0.15-0.4 M NaCl) in Q-buffer. Nine ml fractions were collected. Both chromatographies were carried out on a Pharmacia FPLC system at room temperature.

Refold of sulfonated TFPI

The sulfonated, full-length TFPI pool from anion-exchange chromatography was diluted to an absorbance of 0.07 O.D. units at 280 nm with Q-buffer containing 0.3 M NaCl. Solid L-cysteine was added to a final concentration of 2 mM. The solution was incubated at room temperature for 24 hours, diluted 1:1 with water, 1 mM L-cysteine was added, incubated at room temperature for another 24 hours and then incubated at 4°C for up to 4 to 8 days. pH was maintained at 8.5 by addition of 50 mM Tris.

Mono S Chromatography of refold mixture

In analytical runs, 2 ml refold mixture was loaded onto a Mono S HR 5/5 cation exchange column pre-equilibrated in S-buffer (20 mM sodium phosphate, pH 6.4, 6 M urea). The column was washed with 10 ml of the equilibration buffer and

eluted with a 70-ml gradient consisting of 0-0.7 M NaCl in S-buffer. One-ml fractions were collected. In preparative runs, the refold mixture was acidified to pH 4.5, concentrated 75-fold, and loaded onto a Mono S HR10/16 anion exchange column pre-equilibrated in S-buffer containing 0.3 M NaCl. The column was washed with 15-column volumes of the equilibration buffer and eluted with a 0.3-0.5 M NaCl gradient in S-buffer.

Tissue factor-induced coagulation time assay

Conventional coagulation time assay was performed using a Fibrometer (Becton Dickinson) clot timer. Ninety µl of human pooled plasma was mixed with 10 µl of TFPI sample or control buffer in the well at 37°C for 1 min and 0.2 ml of tissue factor (Simplastin Excel, diluted 1:60 into a solution containing 75 mM NaCl, 12.5 mM $CaCl_2$, and 0.5 mg/ml bovine serum albumin) was added to initiate the clotting reaction.

Amidolytic assay of factor X_a inhibitory activity

Inhibitory activity against bovine factor X_a of TFPI samples were assayed by conventional amidolysis of Spectrozme X_a as described previously by Wun et al., J. Biol. Chem. 265, 16096-16101 (1990) except that the assay buffer consisted of 0.1 M Tris/HCl, pH 8.4, and 0.1% Triton X-100 non-ionic surfactant.

Protein determination

The concentration of protein was determined by absorbance at 280 nm and by quantitative amino acid analysis after HCl/vapor phase hydrolysis at 110°C for 24 hours.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Daiichi precasted 10-20% gradient gels were used for SDS-PAGE. Samples are either unreduced and not boiled or reduced in 3.3% 2-mercaptoethanol and boiled for 3 minutes before electrophoresis. The gels were stained by Coomassie blue.

Expression of TFPI in E. coli

Three vectors were constructed and used for expression of TFPI in E. coli. The first construct, pMON9308, which contained the original human TFPI cDNA sequence (except the initiating ATG) and the rec A promoter, achieved a very low level of expression (<0.5% of total cell protein). The second construct, pMON6870, which was similar to the first but was altered by introducing an alanine at the second position, by increasing the A-T richness of the 5'-end and by improving E. coli codon usage, did not significantly raise the expression level. The third construct pMON6875, which was similar to the second but used a tac promoter, achieved an expression level of approximately 5-10% of total cell protein and was used for further tests herein. The majority of TFPI (>90%) appeared to be sequestered in inclusion bodies.

Sulfonation of inclusion body and purification of full-length sulfonated TFPI

In initial tests, it was found that the E. coli lysate or the isolated inclusion bodies contained very little TFPI activity as measured by anti-factor X_a and by tissue factor-induced coagulation time assays. Refolding of TFPI by reduction/re-oxidation and by sulfonation/disulfide interchange of the crude, solubilized inclusion bodies resulted in very low recovery of activity. Therefore, attempts were made to purify TFPI prior to refolding step, by sulfonation followed by anion

exchange chromatography, taking advantage of the 18 added negatively charged groups on the sulfonated TFPI. The sulfonated inclusion bodies were first fractionated on an analytical Mono Q HR5/5 anion exchange column. The flow-through and early gradient fractions contained much of the contaminants E. coli protein and truncated TFPI protein (the latter are lower in molecular weight and are immuno-reactive against anti-TFPI-Ig). The full-length TFPI-S-sulfonate eluted at about 0.28 M NaCl. The fractionation of sulfonated inclusion bodies was scaled up 20 times using a Hiload Q Sepharose 16/10 anion exchanger. The chromatogram appeared somewhat different from that from Mono Q but the fractionation of the full-length TFPI-S-sulfonate appeared comparable as judged from SDS-PAGE.

Refold of TFPI-S-sulfonate

Sulfonated TFPI underwent spontaneous refolding and oxidation upon mixing with a suitable concentration of L-cysteine. The efficiency of refold as reflected in the increase of TFPI activity varies widely depending on the refold conditions. Numerous refold conditions were compared and optimized in terms of temperature, pH, urea, L-cysteine and protein concentration. A 2-stage refold process appeared to be the best. In the first stage, the full-length TFPI-S-sulfonate pool was adjusted to an absorbance at 280 nm of 0.07 O.D. units, 2 mM of fresh L-cysteine was added, and the mixture was incubated at room temperature for 24 hours. During this period, the TFPI activity increased from 0 to about 12% of full-length SK hepatoma TFPI which served as a standard for comparison. In the second stage, the solution was diluted 1:1 with water, and fresh L-cysteine was added to a final concentration of 1 mM. The mixture was incubated at room temperature for 24 hours, during which time the specific activity increased about 2 fold to about 30% that of SK Hepatoma TFPI. The solution was then left at 4°C for several days during which time the TFPI activity increased.

Fractionation of refold mixture by Mono S chromatography

The specific activity of the refold mixture was lower than the purified mammalian SK TFPI which suggests that the former may contain both correctly folded and misfolded molecules or only partially active misfolded molecules. The refold mixture was fractionated on an analytical Mono S cation exchange column. When the UV-absorbing fractions were analyzed for TFPI activity, the highest specific activity was associated with a sharp peak (fraction 52) eluted at 0.52 M NaCl. All the other fractions had a specific activity less than 30% that of fraction 52. SDS-PAGE analysis showed that fraction 52 contained a sharp band and all other fractions, together with pre-column refold mixture, consisted of diffuse, multiple bands under nonreducing condition. The diffuse bands are apparently mainly full-length TFPI in various folded forms since they become sharp-banded upon reduction (see the last two lanes on the right). By making the gradient more shallow, the resolution of the peaks become better and all the protein peaks appeared to elute at lower NaCl concentrations Further, it was possible to wash out the majority of the low-activity peaks with 10 column volumes of 0.3 M NaCl before eluting the active peak with a shadow gradient.

Based on the above results, the chromatography was scaled up using a Mono S HR10/16 cation exchange column. The column was washed with 15 column volumes of 0.3 M NaCl which essentially washed out all low activity peaks. Afterwards, a shadow gradient eluted a peak of protein that contained the active TFPI. SDS-PAGE analysis shows that the peak gave a sharp band under either reducing or non-reducing conditions. The reduced and boiled protein migrated somewhat slower in SDS-PAGE.

Stoichiometry of the interaction of refolded TFPI with factor X_a

Inhibition of bovine factor X_a by the active refolded E. coli TFPI was examined by measuring the residual amidolytic activity using Spectrozyme X_a. The molar ratio of TFPI to bovine factor X_a that resulted in the complete inhibition of the latter was 1:1 (open circle). For comparison, the stoichiometry of interaction of SK Hepatoma TFPI with bovine factor X_a was also 1:1 (closed circle).

Inhibition of tissue factor-induced coagulation

The ability of the active, refolded E. coli TFPI to inhibit tissue factor-induced coagulation in human plasma was compared with that of the purified SK Hepatoma TFPI. The activity of the E. coli TFPI was approximately two fold more active than SK Hepatoma TFPI on a per mol basis as judged from the concentrations of each TFPI that produce the same prolongation of clotting time.

TABLE 1

Summary of refold and purification of active E. coli TFPI.

	A_{280} nm	Volume (ml)	Total A_{280} nm	Specific activity[a] (SK unit/mA)	Yield (%)
Starting material 0.56 g inclusion body	-	-	-	-	-
Sulfonated inclusion body	6.1	25	153	0	-
HiLoad Q pool	0.8	46	37	0	-
Refold mixture	0.035	1050	37	0.66	100
Mono S pool	0.142	48	6.8	2.0	18

[a] Specific activity was determined by tissue factor-induced coagulation time assay as described in METHODS. One SK unit is defined as the amount of activity equivalent to that produced by 1mA ($1x10^{-3}$ absorbance unit at 280 nm or 1.31 ug) of purified full-length SK hepatoma TFPI.

EXAMPLE 2

This example illustrates the effectiveness of using LACI to treat patients susceptible to or afflicted with sepsis. In particular, this example illustrates the effectiveness of using LACI to treat a sepsis-associated coagulation disorder, namely, DIC.

Recombinant TFPI was expressed as a glycosylated protein using mouse C127 cells as host and was purified by chromatography on a monoclonal antibody convalently attached to Sepharose 4B as described by Day et al. [Blood 76, 1538(1990)].

Baboons, 9 month of age weighing 9-13.9 kg, were randomly selected for LACI or excipient pretreatment (1 hr or 15 min) protocol. Each baboon is immobilized with ketamine hydrochloride, 14 mg/kg intramuscularly on the morning of the study and slowly anesthetized with sodium pentobarbital (~9 mg/kg) via a percutaneous catheter positioned in the cephalic vein and brachial vein. The femoral artery and one femoral vein are cannulated aseptically to measure aortic pressure, obtain blood samples, and for infusion of LACI, live organisms, isotonic sodium chloride and sodium pentobarbital. Animals were pretreated with LACI [3.5 mg LACI per ml of excipient (150 mM sodium chloride and 20 mM sodium sulfate)] or excipient control as an I.V. bolus 40 µg/kg over 15 minutes and then as an infusion at 5.6 µg/kg/min for 545 minutes in the left cephalic vein. Baboons were challenged at time 0 with either 3 ml/kg ($4x10^{10}$) or 4 ml/kg ($5x10^{10}$) of live E. coli. The actual dosing schedule and group assignment appear below:

Group	# of Animals Males	Females	Time of Test Article Administration (min.)	Average Bacterial Dose/ cfu/kg
1	1	4	Excipient -60(3) -15(2)	4.1×10^{10}
2	1	2	LACI -60(3)	3.8×10^{10}
3	0	2	Excipient -15(2)	5.4×10^{10}
4	0	3	LACI -15(3)	4.9×10^{10}

Blood samples were collected at -60 or -15, 0, +60, +120, +240, +360, +600, and +720 minutes for determination of levels of fibrinogen and fibrin degradation products. Results are shown in Tables 2 and 3.

TABLE 2

Individual Animal Fibrinogen Level (% of Time Zero)

	-60/-15 (min.)	0	+60	+120 (min.)	+240 (min.)	+360 (min.)	+720
Group 1	100	100	100	67	40	23	16
	100	100	93	85	16	1	1
	100	95	100	68	32	25	15
	100	100	100	100	29	8	6
	100	100	85	64	52	24	20
Average	100.0	99.0	95.6	76.8	33.8	16.2	11.6
STD DEV	0.0	2.0	6.0	13.7	11.9	9.8	7.0
Group 2	100	84	84	64	84	84	84
	100	100	86	100	86	108	86
	100	83	108	108	100	95	108
Average	100.0	89.0	92.7	90.7	90.0	95.7	92.7
STD DEV	0.0	7.8	10.9	19.1	7.1	9.8	10.9
Group 3	100	100	100	100	44	15	8
	100	100	100	82	19	7	6
Average	100.0	100.0	100.0	91.0	31.5	11.0	7.0
STD DEV	0.0	0.0	0.0	9.0	12.5	4.0	1.0

TABLE 2 (cont.)

Individual Animal Fibrinogen Level (% of Time Zero)

	-60/-15	0	+60	+120 (min.)	+240 (min.)	+360 (min.)	+720 (min.)
Group 4	100	100	121	121	100	100	83
	100	90	90	85	79	62	60
	100	80	91	80	72	75	64
Average	100.0	90.0	100.7	95.3	83.7	79.0	69.0
STD DEV	0.0	8.2	14.4	18.3	11.9	15.8	10.0

Group 1 = Excipient control (4.1 x 10^{10} cfu/kg)
Group 2 = LACI (3.8 x 10^{10} cfu/kg)
Group 3 = Excipient control (5.4 x 10^{10} cfu/kg)
Group 4 = LACI (4.9 x 10^{10} cfu/kg)

TABLE 3

Individual Animal Fibrin Degradation Products (µg/ml)

	-60/-15	+240 (min.)	+720 (min.)
Group 1	10.00	320.00	320.00
	10.00	80.00	320.00
	10.00	80.00	160.00
	10.00	80.00	160.00
	10.00	10.00	160.00
Average	10.00	114.00	224.00
STD DEV	0.00	106.51	78.38
Group 2	10.00	10.00	10.00
	10.00	10.00	10.00
	10.00	10.00	10.00
Average	10.00	10.00	10.00
STD DEV	0.00	0.00	0.00
Group 3	10.00	40.00	160.00
	10.00	20.00	160.00
Average	10.00	30.00	160.00
STD DEV	0.00	10.00	0.00
Group 4	10.00	10.00	20.00
	10.00	10.00	80.00
	10.00	20.00	40.00
Average	10.00	13.33	46.67
STD DEV	0.00	4.71	24.94

Group 1 = Excipient control (4.1×10^{10} cfu/kg)

Group 2 = LACI (3.8×10^{10} cfu/kg)

Group 3 = Excipient control (5.4×10^{10} cfu/kg)

Group 4 = LACI (4.9×10^{10} cfu/kg)

There was a clear effect by LACI on fibrinogen levels in the E. coli treated animals. A drop in fibrinogen is prominent in the excipient controls (Groups 1 and 3) from 240 minutes (i.e., two hours after the end of bacterial infusion) and on. The drop was substantially prevented by LACI pretreatment when the baboons were challenged with lower dose bacteria (Group 2), and attenuated when the animals are challenged with the higher dose bacteria (Group 4).

The generation of fibrin degradation products was not detectable in Group 2, and slowed down and reduced in Group 4 animals as a result of pretreatment with LACI. The differences in the above coagulation parameters among the groups are not as prominent at 720 minutes possibly due to the fact that the LACI infusion was stopped at 540 minutes and that a certain circulating level of LACI may be necessary to maintain an effect.

In addition to the above analyses, histopathology studies wherein tissues of all groups of the above baboons were processed, stained with hematoxylin and eosin, and examined by light microscopy. The kidneys, lungs, adrenals, liver and spleen appeared to be the main organs affected by the E. coli challenge. Reduced pathology in some target organs such as adrenals and kidneys was observed.

Thus, the conclusion drawn from the above is that the effect of LACI on septic shock is evident, particularly in view of the attenuation of the fibrinogen drop end generation of fibrin degradation products, and the reduced pathology in some target organs, such as the adrenal and kidney.

EXAMPLE 3

This example illustrates the effectiveness of using LACI to promote survival in patients which are susceptible to or afflicted with sepsis. In particular, this example illustrates

the effectiveness of using LACI to treat gram-negative sepsis. LACI was prepared by the method described above in Example 1.

Male and female Papio anubis baboons (7.6 ± 2.4 kg) from the Charles River Primate Center (Wilmington, MA) were quarantined for a minimum of thirty days in the University of Oklahoma Animal Facility (Oklahoma City, OK).

Each baboon was immobilized with ketamine hydrochloride, 14 mg/kg intramuscularly on the morning of the study and slowly anesthetized with sodium pentobarbital (-9 mg/kg) via a percutaneous catheter positioned in the cephalic vein. To compensate for insensible fluid loss, the animals were infused with isotonic saline at a rate of 3.3 ml/kg/hr for 12 hours via the barachial vein in the right leg. LACI or PBS buffer control was administered to the animals through the brachial vein 30 minutes after the administration of bacteria. LACI was administered at a loading dose over fifteen minutes and simultaneously started a continuous infusion of LACI for an additional 675 minutes (counting from start of bacterial infusion, which was defined as time zero).

E. coli 086: K61H were used to inoculate tryptic soy broth agar (2); viability counts of the inoculum were determined by standard dilution techniques. At time zero, baboons received an infusion of 4.5×10^{10} live bacteria per kg body weight (4 mls/kg), administered through a percutaneous catheter in the right cephalic vein by continuous infusion for 2 hours.

The femoral artery and one femoral vein were cannulated aseptically to measure mean systemic arterial pressure, obtain blood samples and for antibiotic administaration. Gentamicin was given (9 mg/kg i.v.) at end of E. coli infusion, i.e., at T + 120 min. for 30 minutes and then 4.5 mg/kg at T + 300 min. and T + 540 minutes for 30 min. Gentamicin (4.5 mg/kg IM) was then given at the end of the experiment and once daily for 3 days.

Animals were maintained under anaesthesia and monitored continuously for 12 hours. Blood samples were collected hourly for hematology, clinical chemistry, cytokines (TNF, IL-6) and LACI determinations. Similarly, respiration rate, heart rate, mean systemic arterial pressure and temperature were monitored hourly.

Animals surviving 7 days were considered survivors and subsequently euthanized for necropsy at the 8th day.

See Hinshaw, L.B., Archer, L.T., Beller-Todd, B.K., Coalson, J.J., Flournoy, D.J., Passey, R., Benjamin, B., White, G.L. Survival of primates in LD_{100} septic shock following steriod/antibiotic therapy, J. Surg. Res., 26, 151-170 (1989), and Hinshaw, L.B., Brackett, D.J., Archer, L.T., Beller, B.K., Wilson, M.F. Detection of the "hyperdynamic state" of sepsis in the baboon during lethal E. coli infusion, J.Trauma, 23, 361-365, (1982); which are incorporated herein by reference.

Results of this study are shown in Table 1.

TABLE 1

E. coli LACI Baboon Data

Baboon #	Fibrinogen 240' % of T=0	Fibrinogen 720' % of T=0	Platelet Ct. 720' % of T=0	Hemolysis 6hr	Hemolysis 12hr	Blood Pressure 3hr % of T=0	Blood Pressure 12hr % of T=0	Recovery at 24 hr. Consciousness	Alertness	Mobility	Survival Time
Controls											
3	<1	<1	27	--	--	61	41				18 hrs
6	8	<1	21	-	-	51	84	--	--	--	33 1/2 hrs
18	4	<1	19	-	-	65	84	-	-	-	66 hrs
19	<1	<1	22	--	--	89	122	-	-	-	7 days
20	4	<1	26	+	+	53	45	-	-	-	83 hrs
Low Dose (Loading Dose 0.7 mg/kg; Maintenance Dose 3.0 ug/kg/min)											
7	78	8	37.5	-	--	62	50				18 hrs
8	55	50	32.2	-	-	38	69	++	++	++	53 hrs
10	32	10	15	-	-	73	63	+	+	-	7 days
11	93	83	26	+	+	63	89	++	++	-	7 days
16	43	43	33	-	-	75	80	-	-	-	7 days
17	74	84	30	+	+	75	90	++	+	+	7 days
High Dose (Loading Dose 1.0 mg/kg; Maintenance Dose 9.5 ug/kg/min)											
4	77	22	70	+	+	56	87	+	+	+	7 days
5	79	113	59	+	+	75	77	++	++	++	7 days
12	116	71	48	+	+	79	68	++	++	++	59.5 hrs
13	86	49	30	+	+	61	82	++	++	++	7 days
14	88	105	30	+	+	74	71	++	++	++	7 days
15	54	54	41	+	+	78	90	++	++	++	7 days

Recovery Code

++ Very alert, very mobile
+ Somewhat alert, slightly mobile
- Appears tired, cognizant of surrounding, sitting up, petechia noted
-- Lying down, not cognizant of surroundings, eyes blinking, petechia noted

Hemolysis Code

- Hemoylsis noted
+ No hemoylsis noted

Total daily dose administered to a host in single or divided doses may be in amounts, for example, from about 2 to about 50 mg/kg body weight daily and more usually 4 to 20 mg/kg, preferably, from about 6 to about 10 mg/kg. Dosage unit compositions may contain such amounts of submultiples thereof to make up the daily dose.

The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the patient treated and the particular mode of administration.

The dosage regimen is selected in accordance with a variety of factors, including the type, age, weight, sex, diet and medical condition of the patient, the severity of the condition, the route of administration, pharmacological considerations such as the activity, efficacy, pharmacokinetic and toxicology profiles, whether a drug delivery system is utilized and whether the compound is administered as part of a drug combination. Thus, the dosage regimen actually employed may vary widely and therefore may deviate from the preferred dosage regimen set forth above.

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in water. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. A preferred injectable preparation

solution is LACI in an aqueous solution of 150 mM sodium chloride and 20 mM sodium phosphate.

While LACI can be administered as the sole active anticoagulation pharmaceutical agent, it can also be used in combination with one or more antibodies useful for treating sepsis, such as, for example, anti-endotoxin, monoclonal antibodies (endotoxin-binding Mabs) and anti-TNF products such as an anti-TNF murine Mab. LACI can also be combined with interleukin-1 receptor antagonists, bactericidal/permeability increasing (BPI) protein, immunostimulant, compounds having anti-inflammatory activity, such as PAF antagonists and cell adhesion blockers. When administered as a combination, the therapeutic agents can be formulated as separate compositions which are given at the same time or different times, or the therapeutic agents can be given as a single composition.

The foregoing is merely illustrative of the invention and is not intended to limit the invention to specific examples. Variations and changes which are obvious to one skilled in the art are intended to be within the scope and nature of the invention which are defined in the appended claims.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

What is Claimed is:

1. A method of using LACI comprising administering to a patient afflicted with or susceptible to sepsis a therapeutically-effective amount of a composition comprising LACI, said composition being essentially free of heparin.

2. A method of treating sepsis comprising administering to a patient in need thereof a therapeutically-effective amount of a composition comprising LACI, said composition being essentially free of heparin.

3. A method of treating a sepsis-associated coagulation disorder comprising administering to a patient in need thereof a therapeutically-effective amount of a composition comprising LACI, said composition being essentially free of heparin.

4. The method of Claim 3 wherein said patient is afflicted with sepsis.

5. A method of Claim 3 wherein said sepsis-associated coagulation disorder is DIC.

6. A method of preventing a sepsis-associated coagulation disorder comprising administering to a patient in need thereof a therapeutically-effective amount of a composition comprising LACI, said composition being essentially free of heparin.

7. The method of Claim 6 wherein said patient is susceptible to sepsis.

8. A method of preventing sepsis-associated DIC comprising administering to a patient in need thereof a therapeutically-effective amount of a composition comprising LACI, said composition being essentially free of heparin.

9. The method of Claim 8 wherein said patient is susceptible to sepsis.

10. A method of treating a patient afflicted with or susceptible to sepsis comprising administering to a patient in need thereof a therapeutically-effective amount of a composition comprising LACI, said composition being essentially free of heparin.

11. A method of promoting survival in a patient afflicted with or susceptible to sepsis said method comprising administering to a patient in need thereof a therapeutically-effective amount of LACI.

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K37/64; //C12N15/15

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	FILE SERVER STN KARLSRUHE,FILE MEDLINE ABSTRACT NO.92135323 & BLOOD COAGUL. FIBRINOLYSIS (1990 OCT.), 1 (4-5),415-26 BROZNA:'CELLULAR REGULATION OF TISSUE FACTOR' see abstract ---	1-11
A	FILE SERVER STN KARLSRUHE,FILE MEDLINE ABSTRACT NO.91356022 & BLOOD (1991 SEP.15), 78 (6),1496-502 SANDSET ET AL:'IMMUNODEPLETION OF EXTRINSIC PATHWAY INHIBITOR SENSITIZES RABBITS TO ENDOTOXIN-INDUCED INTRAVASCULAR COAGULATION AND THE GENERALIZED SHWARTZMAN REACTION' see abstract --- -/--	1-11

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 SEPTEMBER 1993	29 -09- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	SITCH W.D.C.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	FILE SERVER STN KARLSRUHE,FILE MEDLINE ABSTRACT NO.89323492 & BLOOD (1989 AUG.15), 74 (3), 994-8 WARR ET AL:'HUMAN PLASMA EXTRINSIC PATHWAY INHIBITOR ACTIVITY:II.PLASMA LEVELS IN DISSEMINATED INTRAVASCULAR COAGULATION AND HEPATOCELLULAR DISEASE' see abstract	1-11
A	WO,A,9 119 514 (NOVO NORDISK A/S) 26 December 1991 cited in the application see the whole document	1-11
P,X	FILE SERVER STN KARLSRUHE,FILE MEDLINE ABSTRACT NO.93294027 &J.CLIN.INVEST. (1993 JUN.), 91(6),2850-6 CREASEY ET AL:'TISSUE FACTOR PATHWAY INHIBITOR REDUCES MORTALITY FROM ESCHERICHIA COLI SEPTIC SHOCK' see abstract	1-11

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-11 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9305409
SA 75669

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 14/09/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9119514	26-12-91	AU-A- 8060491	07-01-92
		EP-A- 0535129	07-04-93